INFLUENCE OF CYANIDE AND LACK OF OXYGEN ON THE ACTIVATION OF STARFISH EGGS BY ACID, HEAT AND HYPERTONIC SEA-WATER

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In previous papers (Lillie, 1915, 1917, 1926) I have called attention to the general resemblances between the phenomena of acid activation and of heat activation in unfertilized starfish eggs,—a parallelism indicating that both forms of activation are to be referred to the same kind of change in the egg system.[1] This primary or critical change in some way transforms the egg from the quiescent into the automatically developing or "activated" state. Since exposure to temperatures above the physiological range is known to produce acid (especially lactic acid) in many cells (as seen, *e.g.*, in the phenomena of heat rigor), the inference is natural that heat activation is in reality a secondary effect resulting from the production of acid within the egg; and the essential problem becomes that of determining the factors concerned in acid activation. Experiments on the relative activating effectiveness of a variety of weak penetrating acids having a wide range of dissociation constants (Lillie, 1926 and 1927) indicate that the essential factors in a given activating effect are (1) the production of a certain definite degree of acidity in some region of the egg (probably cortical) and (2) the maintenance of this acidity for a definite time. Some progressive change, the "activation process" or "activation reaction," occurs within the egg during this time; and if the egg is to be rendered capable of developing to an advanced stage, the process must advance to a certain stage of completion. One of the most striking features of the activation process in starfish eggs is that it is readily arrested at any stage by simply returning the eggs to normal sea-water; in such a case it may be renewed and brought to completion by a second exposure to heat or acid, applied after not too long an interval (Lillie, 1915, pp. 284 *seq.*). The rate of the activation process, in the case of a particular acid, appears closely proportional to the concentration of

[1] The chief parallels are (1) the similarity in the time-relations of partial and complete activation by the two methods; (2) the fact that both are completely effective only during the prematurational period of the egg; (3) the substitutability of one for the other in experiments on partial activation, and (4) other parallels described in the present paper.

the latter in the external medium—more specifically to the concentration of its undissociated molecules, which alone appear to penetrate freely to the site of the activation reaction (Lillie, 1926 and 1927).

Since apparently any non-toxic penetrating acid can activate starfish eggs, the special chemical nature of the acid would seem to be immaterial; its presence merely enables the activation process to proceed in the egg system. In general our present data indicate that the rate of activation is closely proportional to the acidity (cH above a certain critical level) attained at the site of activation. The most reasonable assumption appears to be that activation is an effect of the chemical union of certain specific substances already present or available in the egg,[2] and that this interaction, the primary or key reaction in the activating sequence, occurs only above a certain level of acidity (*i.e.*, within a certain pH range). The implication is that the product of this reaction—which we may call the "activating substance"—requires to accumulate to a certain definite level if complete activation is to result. Eggs in which this critical quantity of activating substance has been formed proceed normally with their development if placed under appropriate external conditions. That the accumulation of some material is a necessary condition of activation is indicated by the progressive character of the activation reaction, and especially by the possibility (already referred to) of arresting it at an incomplete stage and afterwards renewing it.

The activation reaction cannot be characterized definitely in chemical terms at present. The fact that responsiveness to activation, either by parthenogenetic agents or sperm, begins normally to decline at or about the time of separation of the first polar body and remains incomplete during the post-maturational period indicates a loss or destruction of some reactant or reactants at this time.[3] Completely mature eggs are capable of only partial activation: although such eggs still form fertilization-membranes and cleave after treatment with fatty acids or warm sea-water, only a small proportion (if any) develop to swimming stages; similarly only a minority of eggs fertilized with sperm during this period exhibit normal development. The capacity for complete activation is lost, apparently permanently, during the progress of the maturation divisions (Lillie, 1908).

[2] This is also the assumption of the fertilizin hypothesis of F. R. Lillie, which is quite consistent with the present view. *Cf.* Lillie, F. R.: "Problems of Fertilization," University of Chicago Press, 1919; also E. E. Just: "The Present Status of the Fertilizin Theory of Fertilization." *Protoplasma*, 1930, Vol. 10, p. 300.

[3] The term "prematurational period" is used in the present paper to designate the period between the dissolution of the germinal vesicle and the separation of the first polar body; the post-maturational period is that succeeding the separation of the second polar body.

This change in the physiological properties of the egg, like the change leading to its natural death if it is left unfertilized, (Loeb, 1902 *a* and *b*) is intimately dependent on normal respiration, as is shown experimentally by the effects of cyanide or deprivation of oxygen. If freshly removed starfish eggs are placed for two to four hours (at 20°) in sea-water freed of oxygen or containing KCN (M/500, M/1000), and are then returned to normal sea-water, they show an almost unimpaired response to fertilization or artificial activation; while the response of control eggs left for the same interval in normal sea-water is partial or defective. Such facts indicate that some material essential to the activation reaction (*i.e.*, some precursor or precursors) is removed or destroyed at a definite stage in the normal oxidative metabolism of the egg; apparently this occurs most rapidly during the period at which the polar bodies are being separated.

A further fact of significance is that the activation reaction, as such (*i.e.*, the reaction occurring during the actual exposure to fatty acid or heat), is independent of immediate oxygen consumption; that is, the eggs respond in a normal manner to heat or fatty acid while immersed in oxygen-free or cyanide-containing sea-water—even if they have previously been exposed for some hours to these media. Such suppression of oxidative metabolism has, however, a characteristic modifying influence on the rate of activation, as is shown by the experiments to be described below.

1. *Influence of Oxygen-lack or Cyanide on the Effect of a Second Activating Treatment Following Partial Activation by a First Treatment*

It was found in earlier experiments (Lillie, 1915, pp. 284 *seq.*) that two successive brief exposures to either heat or fatty acid, separated by a considerable interval (up to 30 minutes or more), may by a process of summation result in complete activation, although either exposure acting alone has only a partial effect.[4] A single continuous exposure lasting as long as the sum of the other two also causes complete activation. Such an experiment shows that the modification (whatever its nature) produced by the first exposure persists for some time, and that the second exposure induces further modification of the same kind. If the first exposure is made early, *e.g.*, within 5 to 10 minutes after the

[4] In partial activation the eggs form typical fertilization-membranes, but cleave slowly and irregularly (if at all) and die before reaching the blastula stage. In complete activation cleavage approaches the normal in rate and regularity and the great majority (in favorable cases all) of the eggs form active blastulæ and gastrulæ. All gradations in degree of activation can be obtained by varying the length of exposure.

commencing dissolution of the germinal vesicle, an hour or more may elapse without marked decline in the effect of the second exposure; later this exposure becomes progressively less and less effective. This decline in the response to a second activating treatment appears to follow the same course as the normal decline of activability already described. It also depends on the oxidative metabolism of the egg and can be greatly retarded by cyanide or removal of oxygen; apparently it is referable to a depletion of the reserve of activable material (precursor or precursors of the activating substance) left unchanged by the first treatment.

The following experiment (June 10, 1927) will illustrate. The mixed eggs from three starfish were exposed during the prematurational period for 6 minutes to .002 M acetic acid in balanced NaCl—$CaCl_2$ solution at 20° C. Part (*A*) was then transferred to normal sea-water (at *ca.* 18° C.) and part (*B*) to sea-water containing M/1000 KCN. At the intervals indicated in Table I eggs from both lots were again exposed for 6 minutes to the same acid solution and returned to normal sea-water. The total exposure, 12 minutes, is approximately the optimum for this solution at 20°. The intervals between the two successive exposures varied from 11 minutes to two hours. The results are shown in Table I.

TABLE I

Interval between exposures (*minutes*)	Percentage of eggs forming blastulæ as result of second exposure	
	A. Eggs kept in normal sea-water	B. Eggs kept in M/1000 KCN in sea-water
11	90	90
20	60–65	90
30	60–70	90
45	60–70	70–80
60	60–70	80–90
90	50	70–80
120	5	50

Eggs exposed for 6 minutes without any second treatment showed typical partial activation, and a few (2–3 per cent) formed blastulæ. Sperm-fertilized eggs developed normally.

In normal sea-water nearly all the eggs have lost responsiveness to the second treatment after two hours, while of the eggs remaining in cyanide-containing sea-water for the same interval a large proportion developed. Table II describes a second similar experiment (July 11, 1927) with longer intervals between exposures.

TABLE II

July 11, 1927. The procedure was the same as in the previous experiment. The acid solution used was .0015 M acetic acid in Na-Ca solution at 20° C. A control series showed an optimum continuous exposure of 12–14 minutes, yielding 60-70 per cent blastulæ. The first exposure was 6 minutes; eggs so treated without a second exposure gave membranes and some cleavage but no blastulæ. After each interval the eggs kept in sea-water received second exposures of 6, 8, and 10 minutes; those kept in KCN sea-water received 4, 6, 8, and 10 minutes.

Interval between exposures (*hours*)	Percentage of eggs forming blastulæ and optimum durations of second exposure	
	A. Eggs kept in normal sea-water	B. Eggs in M/1000 KCN in sea-water
1	35–40 (8 and 10 min.)	*ca.* 50 (6–8 min.)
3	few (1%) (6–8 min.)	50–65 (4–6 min.)
5	0 (6–8 min.)	25–35 (4–8 min.)
22	(all dead by 22 hrs.)	0

Sperm-fertilized eggs developed normally.

Well-marked response to the second exposure after 5 hours in M/1000 KCN was observed in four other similar experiments and also in three experiments in which the eggs were left for 5 hours in oxygen-free sea-water between exposures. Four experiments with periods of 18 to 22 hours in M/1000 KCN showed almost no response after this interval, although in one experiment some slight effect was apparent after 19 hours.

Sea-water deprived of oxygen (by prolonged boiling, restoration of the original volume with boiled distilled water, and passage of a stream of hydrogen or nitrogen overnight) prolongs similarly the possible summation-interval. For example, on August 16, 1927, eggs kept for 2 1/2 and 5 hours in oxygen-free sea-water, after a first exposure of 5 minutes to .002 M acetic acid, yielded with both intervals 60–70 per cent of blastulæ as the result of a second exposure of 6 to 8 minutes to the same solution. The control eggs kept in normal sea-water showed almost no response to the second exposure. The eggs which received only the first exposure of 5 minutes showed typical partial activation with no blastulæ, while those receiving continuous exposures of 12, 13 and 14 minutes at the same time (30 minutes after removal from the animals) gave 75–90 per cent of blastulæ.

Seventeen experiments of this kind were performed during the summer of 1927 with results similar to those cited. In all cases the interval between the first inadequate and the second effective exposure was prolonged to several hours by the suppression of oxidative metabolism in the manner indicated.

These results are in conformity with the hypothesis that some material essential to the production of the activating substance disappears normally from the egg in the course of the regular oxidative metabolism. Hence the unused reserve of this material is retained within the egg for a longer period, together with the activating substance formed by the first exposure, if oxygen-consumption is prevented.

There is, however, some indication that the reserve of precursor material (activable substance) remaining in the egg after the first exposure is not retained entirely unaltered during the interval, but that it undergoes gradual transformation into activating substance. This is seen in the fact that partially activated eggs show an increase in the degree of their activation (as compared with eggs of the same lot left in normal sea-water), if they are kept undisturbed for some hours in oxygen-free or cyanide-containing sea-water. For example, in the experiment just cited (August 16, 1927) eggs left for two hours in oxygen-free sea-water (after the initial exposure of 5 minutes to .002 M acetic acid), and then transferred to normal sea-water, yielded without any second treatment 10–15 per cent of blastulæ, while after remaining for 5 hours in oxygen-free sea-water more than 50 per cent formed blastulæ. In the latter case (5 hours without oxygen) a second exposure of two minutes proved almost as effective as one of 6 minutes, both yielding 65–70 per cent of blastulæ. Such cases show that a partial activation may be completed in many eggs by simple exposure of one to several hours to cyanide-containing sea-water. This effect is similar to Loeb's completion of activation in sea-urchin eggs, after membrane-formation by acid, by after-treatment with cyanide-containing sea-water (Loeb, 1913). In starfish eggs a well-marked shortening of the optimal duration of the second acid treatment was a constant effect of one or more hours exposure to oxygen-free or cyanide-containing sea-water. Apparently prolonged suppression of oxygen-consumption in the partially activated eggs has a physiological effect similar to that of treatment with acid or high temperature.

It seems probable that the production of acid within the egg under the conditions of anaërobic metabolism is responsible for the increase of activation shown in such cases. This supposition is consistent with the fact that previous exposure of normal untreated starfish eggs to oxygen-free or cyanide-containing sea-water also very definitely increases the susceptibility to activation by either heat or acid, as will be shown in the next section. I have, however, never succeeded in activating such eggs, even to the extent of membrane-formation, by this treatment alone.[5] The acid formed under these conditions may

[5] E. P. Lyon obtained parthenogenetic development in the *Strongylocentrotus* egg at Naples by prolonged exposure to solutions of KCN (M/100 to M/1000) in sea-water (*cf.*, *Am. Jour. Physiol.*, 1903, Vol. 9, p. 308).

not reach the critical level of concentration required for activation, or it may be ineffective for some independent reason.

2. *Activation after Previous Exposure of Normal Eggs to Cyanide and Lack of Oxygen*

In the experiments just described partially activated eggs after remaining some hours in oxygen-free or cyanide-containing sea-water showed a tendency to complete their activation spontaneously; *i.e.*, the second effective exposure to acid was unexpectedly brief, and many eggs formed blastulæ without any second exposure. Similarly, normal unactivated eggs kept under the same oxidation-suppressing conditions show in course of time an increasing susceptibility to activation by either heat or acid. Suppression of oxygen-consumption has thus a double effect on the eggs: (1) prolongation of the time during which they remained activable, and (2) increase of activability, as shown by a decrease in the effective durations of exposure to the activating agent.

Cyanide-containing Sea-water.—A typical series in which the eggs were exposed to KCN-containing sea-water for 2 1/2 hours before the activating treatment is the following:

July 3, 1929. Eggs from several starfish were placed, 35 minutes after removal, in sea-water containing M/1000 KCN. Two hours, 35 minutes later, a part (*B*) was washed in several changes of normal sea-water, left in sea-water for 26 minutes and then placed in M/260 butyric acid solution in sea-water. A second part (*C*) after 2 hours, 32 minutes in the KCN sea-water was transferred directly to KCN sea-water containing also M/260 butyric acid.[6] The essential difference between *B* and *C* is that in *B* the activating treatment occurred after the eggs were washed free of cyanide, while in *C* the entire period preceding and during activation was spent in the presence of cyanide. At two-minute intervals eggs were transferred from the butyric acid

[6] In estimating the rate of activation in solutions of fatty acid containing KCN, allowance must be made for the neutralizing action of this salt, which is very nearly the same as that of $NaHCO_3$. In general the rate of activation exhibited by starfish eggs placed directly from sea-water in solutions of fatty acid containing also KCN is closely similar to that of eggs exposed to solutions of the same non-neutralized acid strength without KCN. This was shown experimentally as follows: Eggs exposed to balanced $NaCl$-$CaCl_2$ solution containing both acetic acid and KCN in equal concentrations, .002 M, showed no membrane formation or other signs of activation with exposures from 5 to 18 minutes. The control experiment with .002 M acetic acid alone showed optimal activation at 10 minutes, with 75–80 per cent of eggs forming blastulæ. The same $NaCl$-$CaCl_2$ solution containing .002 M acetic acid plus .001 M KCN gave a rate of activation similar to that of .001 M acetic acid without KCN. The solution used above, sea-water containing M/260 butyric acid *plus* M/1000 KCN, corresponds closely in its activating effect on normal eggs to a solution of .003 M butyric acid in sea-water (*i.e.*, M/260, *ca.* .0038–.001 M).

solutions to normal sea-water; later the proportions developing to blastulæ were determined. The control (*A*) consisted of eggs exposed to M/260 butyric acid in sea-water, beginning 40 minutes after removal from the animals.

Table III gives the approximate percentages of eggs forming blastulæ with the different exposures.

TABLE III

Duration of exposure (20° C.) (*minutes*)	Percentage of eggs forming blastulæ		
	A. (Control without KCN exposed to normal sea-water containing M/260 butyric acid)	B. (KCN 2½ hrs. exposed to normal sea-water containing M/260 butyric acid)	C. (KCN 2½ hrs. exposed to sea-water containing M/260 butyric acid *plus* M/1000 KCN)
2	0	40–50	no observation
4	1	30–40	60–70
6	15–20	30–40	60–70
8	30–40	60–70	50
10	*ca.* 90	70–80	20–30
12	80–90	70–80	*ca.* 10
14	70–80	30–35	*ca.* 10
16	20–30	*ca.* 10	1

Nearly half the eggs in Part *B* formed blastulæ with exposures as brief as two minutes; also a large proportion continued to develop favorably with longer exposures up to 12 minutes. The effect is even more striking in Part *C*. Here the effective (non-neutralized) concentration of acid in normal sea-water (*ca.* .003 M) typically requires 20 minutes or more for complete activation; yet after the preliminary treatment with KCN, two-thirds of the eggs formed blastulæ with exposures of 4 to 6 minutes.

Two other series on July 15 and 16, with preliminary exposures of 3 and 5 hours (respectively) to KCN sea-water gave closely similar results. In the series of July 15 eggs exposed to M/260 butyric acid in sea-water, after 3 hours in KCN sea-water, for periods of one to 6 minutes, gave 60–80 per cent blastulæ. With the cyanide-containing butyric solution one minute was ineffective, but exposures of 2 to 6 minutes yielded 50 per cent or more blastulæ. The results of July 16, with 5 hours previous treatment with KCN, were similar; butyric acid in normal sea-water showed optimal activation at 2 minutes with 70–80 per cent blastulæ; the same solution in KCN-containing sea-water was ineffective at 2 minutes, but with exposures of 4 to 14 minutes gave 50 per cent or more blastulæ. On July 17 and 18 similar series with 5

and 3 hours in KCN-sea-water showed the same effect with somewhat fewer blastulæ.

The shortening of the effective exposures under the influence of KCN is appreciable although slight after 15 or 20 minutes and increases with time. Table IV gives the results of a typical series in which the preliminary exposure to KCN was varied from 15 to 65 minutes. The optimum exposure for the control eggs unexposed to KCN was 10 minutes (21° C.), with more than 90 per cent forming blastulæ.

TABLE IV

Experiment of June 20, 1930. Eggs were exposed during the prematurational period to M/260 butyric acid in normal sea-water at 21° C. after washing free of cyanide.

Previous Exposure to M/1000 KCN (*minutes*)	Times of Exposure to Butyric Acid (*minutes*) and Percentages of Eggs Forming Blastulæ								
	2	3	4	5	6	7	8	9	10
None (control)	0	0	0	2–3	2–3	5–10	*ca.* 50	65–75	90
15	0	0	0	2–3	2–3	60–70	60–70	75–85	80–90
26	0	*ca.* 1	*ca.* 1	*ca.* 1	25–35	50–60	70–80	70–80	*ca.* 50
45	1	*ca.* 5	10–20	40–50	60–70	65–75	50–60		
65	*ca.* 1	*ca.* 5	10–15	20–25	35–45	55–65	25–35		

Three other similar series carried out between June 17 and June 23, 1930, with exposures to KCN ranging from 12 minutes to 5 hours, gave the same general result. In general, the degree of shortening increases with duration of exposure up to a maximum of 2 to 3 hours. After 5 hours there is usually a decline of responsiveness, and after 20 to 24 hours no blastulæ were obtained, although there was some partial activation.

Oxygen-free sea-water.—Experiments with eggs remaining for 2 to 5 hours in oxygen-free sea-water before activation with fatty acid gave a similar result.[7] Eight successive series between July 23 and August 3, 1929 showed uniformly a striking decrease in the activating exposures; of such eggs a large proportion and in some cases nearly all formed blastulæ after exposures of one to 2 minutes to M/260 butyric acid. Table V gives a summary of the first four series. The eggs, after exposure to the oxygen-free sea-water (in flasks through which a slow stream of hydrogen was kept flowing) for the periods named, were treated for

[7] In general the physiological effects of exposing starfish eggs to cyanide-containing and to oxygen-free sea-water are strikingly similar. One constant effect following exposure for one to several hours to either medium is to render the eggs glutinous or sticky, so that they cohere in masses, a result (apparently) of the secretion of some water-swelling or glutinous material.

periods ranging from one to 16 minutes with M/260 butyric acid made up in both normal and oxygen-free sea-water. The controls, as before, consisted of eggs exposed during the prematurational period (usually 30 to 45 minutes after removal) to M/260 butyric acid,[8] again both with and without oxygen. These showed optima varying between 6 and 12 minutes (most frequently at 8 minutes) with 70 to 85 per cent of eggs forming blastulæ. The temperatures of the sea-water and butyric acid solutions were 21° to 22.5° C.

TABLE V

Experiment and date (1929)	Control (Effective exposures and percentages of eggs forming blastulæ)		Period in O_2-free sea-water before activation		Exposures to M/260 butyric acid and percentages of resulting blastulæ			
					(a) O_2-containing acid solution		(b) O_2-free acid solution	
	min.	%	*hrs.*	*min.*	*min.*	%	*min.*	%
1. July 23	6	(35–40)	2	15	2	(*ca.* 50)	2	(*ca.* 50)
	8	(70–80)			4	(35–45)	4	(50–60)
	10	(50–60)			6, 8	(25–35)	6	(35–40)
					10	(20–35)	8, 10	(25–35)
							12	(20–25)
2. July 24	4	(25–35)	5		1	(40–50)	1	(35–40)
	6	(80–90)			2, 3	(20–30)	2	(30–35)
	8	(50)			4	(5–10)	3	(15–20)
	10	(5)			6	(0)	4	(*ca.* 1)
3. July 25	6	(40–50)	4	25	1, 2	(80–90)	1	(75–85)
	8	(75–80)			3, 4	(75–85)	2. 3	(80–90)
	10	(*ca.* 90)			5	(70–80)	4, 5	(70–80)
	12	(50–60)			6, 7	(*ca.* 60)	6, 7	(65–75)
					8	(50)	8	(55–60)
4. July 26	8	(10–15)	3	40	2, 4	(70–80)	2, 4	(70–80)
	10	(40–50)			6, 8	(60–70)	6	(60–65)
	12, 14	(70–80)			10	(*ca.* 50)	8	(*ca.* 50)
					12, 14	(25–35)	10	(30–40)
							12	(*ca.* 5)

In three other series (August 1, 2 and 3, 1929) eggs kept previously for equal times in both KCN-containing and oxygen-free sea-water (respectively 2 1/4, 3 and 4 1/2 hours) were exposed to M/260 butyric acid in sea-water. In all cases exposures of 1, 2 and 3 minutes gave numerous blastulæ (60 to 80 per cent in five of the six experiments); exposures of 4, 5 and 6 minutes were less effective, and with 8 minutes

[8] Two cc. M/10 butyric acid *plus* 50 cc. sea-water. Oxygen-free sea-water was used in mixing solution (*b*) and hydrogen was bubbled through the mixture for some hours.

almost no blastulæ were formed. The control eggs gave no blastulæ with the two-minute exposure, few with 4 minutes and a maximum (60–80 per cent) with 6 to 8 minutes.

One uniform feature of these experiments was that the eggs, after prolonged suppression of oxidative metabolism, not only gave complete activation with brief exposures to acid, but also showed a less sharply defined optimal period of exposure; *e.g.*, in the experiments of July 25th and 26th exposures ranging from one to 8 minutes were almost equally effective in producing blastulæ (65 to 85 per cent). This behavior differs strikingly from that of eggs activated in normal sea-water soon after removal; in this case the curve relating percentages of blastulæ to durations of exposure rises rapidly to a maximum—usually at 8 to 10 minutes at this temperature (21°–22° C.) and concentration of acid—and declines equally rapidly, appearing nearly symmetrical.[9] Under the anaërobic conditions just described, over-exposure seems less deleterious; the optimum is reached early, and with further prolongation of the activating treatment there is a more gradual decline of effect; *i.e.*, over-activation is caused less readily, an indication (possibly) of a partial depletion of activable or precursor material during the interval in oxygen-free sea-water. A similar prolongation of the optimum is seen in eggs remaining for some hours in KCN-sea-water previous to activation.

Controls of sperm-fertilized eggs were also kept during these experiments: at the end of the period in oxygen-free or KCN-containing sea-water eggs were transferred to normal sea-water, washed by changing the latter, and immediately fertilized by sperm. In general, responsiveness to sperm fertilization was found to run closely parallel with activability by artificial agents; *i.e.*, normal fertilizability is also prolonged for several hours by exposure to anaërobic conditions. Typically, sperm-fertilization is complete and uniform only during the prematurational period and falls off rapidly with the separation of the polar bodies (Lillie, 1908). Both kinds of prolongation of responsiveness are to be regarded as expressions of the same kind of change in the egg; in terms of the foregoing hypothesis, the precursor material, which is transformed into activating substance under the influence of both acid and sperm, is retarded in its breakdown or removal by the suppression of oxidative metabolism.

3. *Influence of O_2-lack and KCN on Activation by Heat*

The effective exposures to activating temperatures (*e.g.*, 32°) are similarly shortened by previous exposure to cyanide or lack of oxygen.

[9] *Cf.* the curves in my article on activation by acids in *Jour. Gen. Physiol.*, 1926, Vol. 8, p. 339.

In general, however, I have found that in the case of heat activation the decline in responsiveness during such exposure is more rapid than with acid activation. This may indicate that the material which gives rise to the activating acid (presumably carbohydrate) undergoes comparatively rapid depletion with time. In some cases, the eggs have preserved good responsiveness to heat for as long as 3 hours in KCN-containing sea-water, although usually the response is greatly diminished by this time. Apart from this difference the general phenomena are closely similar.

The procedure was the same as in my earlier experiments on heat activation (Lillie, 1908, 1915). The eggs, together with a small quantity of sea-water, were placed in a beaker (or small flask) to which 100–200 cc. of sea-water at the required temperature (32°) was added. The temperature was kept constant by a water bath. At intervals eggs were transferred to a series of dishes containing normal sea-water.

The following experiment (August 6, 1929) is cited as an illustration. One portion of starfish eggs (*A*) was placed, one half hour after removal from the animals, in M/1000 KCN in sea-water; another portion (*B*) in oxygen-free sea-water; in this case one to 2 cc. of a dense egg suspension was added to 100 cc. of oxygen-free sea-water contained in a flask through which hydrogen had flowed overnight, and a slow flow of H_2 was continued. After 3 hours eggs from both lots were treated with normal sea-water at 32° for the times indicated. It had previously been found that eggs left in normal sea-water for 2–3 hours, and then warmed, show only partial response and never form blastulæ (Lillie, 1908).

TABLE VI

Treatment	Percentage of eggs forming blastulæ after exposure to 32° C. for the times indicated (*minutes*)							
	1	2	3	4	5	6	7	8
1. Control: warm sea-water, 30 min. after removal.....	0	1	2–4	25–35	75–85	75–85	60–70	25–35
2. (*A*) In KCN-sea-water 3 hrs., 10 min. before warming.....	10–15	25–35	*ca.* 50	25–35	20–30	1–2	0	0
3. (*B*) In 0_2-free sea-water 3 hrs., 10 min. before warming.....	*ca.* 1	*ca.* 5	20–25	30–35	5–10	1–2	0	0

The preservation of responsiveness and the shortening of the effective exposures are both well shown in this series. In six other similar

experiments during the same season, with exposures of one hour or more to KCN, the effective exposures were similarly decreased, but fewer eggs formed blastulæ. With a briefer previous stay in KCN-containing-sea-water (19 to 24 minutes in five experiments between June 25 and July 1, 1930), the decrease was less marked but still definite. The actual exposure to heat may be made either in normal or in KCN-containing sea-water with the same result. Even without a previous stay in KCN-containing-sea-water, eggs warmed in KCN-containing or oxygen-free sea-water show a distinctly briefer optimal exposure than eggs of the same lot warmed in normal sea-water. Table VII gives a summary of experiments illustrating these effects.

TABLE VII

Procedure	Percentages of eggs forming blastulæ after exposure to 32° for the times indicated (*minutes*)								
	1	2	3	4	5	6	7	8	9
1. June 25, 1930.									
(*a*) Control: normal sea-water at 32°	0 (no membranes)	0 (few membranes)	0 (all form membranes)	0	10–15	10–15	20–25	30–35	*ca.* 10
(*b*) No previous exposure to KCN; KCN-containing sea-water at 32° at same time as control	0 (membranes in nearly all eggs	0	0	10–20	20–30	35–40	5–10	0	
2. June 28, 1930.									
(*a*) Control: normal sea-water at 32°	0 (no membranes)	0 (50% membranes)	0 (all membranes)	*ca.* 1	25–35	60–65	*ca.* 60	10–20	
(*b*) Previous exposure to KCN sea-water for 17 minutes; then direct to KCN-sea-water at 32°	0 (all form membranes)	0	5–10	15–25	60–70	20–30	1	0	

Lack of oxygen has the same effect as KCN upon the rate of heat activation. One uniform and readily observed effect is the shortening of the exposures required for membrane-formation. Typically eggs exposed to normal sea-water at 32° for one minute do not form mem-

branes or show any other signs of activation; exposures of 2 1/2 to 3 minutes are required to form membranes in a majority of eggs. But if eggs are warmed in KCN-containing or O_2-free sea-water (especially after previous exposure to either medium) for one minute, all (or nearly all) form membranes (*cf.* Table VII). Restoration of normal oxygen-consumption reverses this sensitivity,—*i.e.*, eggs exposed as above to oxygen-free or cyanide-containing sea-water and then returned for a few minutes to normal sea-water are found to require 2 or 3 minutes at 32° for membrane-formation, like previously untreated eggs.

Membrane-formation is an index of partial activation and is a regular effect of brief exposure to either heat or acid, as well as to other parthenogenetic agents such as solutions of cytolytic compounds and pure isotonic alkali salt solutions. Complete activation, however, is obtained only with heat and acid,[10] but with these agents the exposures required for the formation of blastulæ are several times longer than those required for membrane-formation alone. In confirmation of the hypothesis that heat acts indirectly through the intra-cellular production of acid, the following experiment is cited. Eggs were exposed to neutral, acid and alkaline artificial unbuffered sea-water (van't Hoff's solution) [11] at 34° as indicated in Table VIII.

TABLE VIII

Solution in which eggs were warmed (34°)	Times of exposure (*minutes*) and percentages of resulting blastulæ								
	1	1½	2	2½	3	3½	4	4½	5
1. Neutral van't Hoff	0 (*ca.* 50% membranes)	2–3	20–30	65–75	50–60	*ca.* 50	15–20	10–15	1
2. van't Hoff *plus* N/1000 NaOH	0 (40–50% membranes)	*ca.* 5	30–40	*ca.* 90	*ca.* 90	75–85	20–30	1–2	0
3. van't Hoff *plus* N/1000 HCl	25–30 (*ca.* 100% membranes)	55–65	75–85	20–30	1	0	0	0	0

Neutral or moderately alkaline balanced isotonic salt solution at 34° has the same action as sea-water at this temperature, while acidulation to this degree nearly doubles the rate of activation. This is

[10] *I.e.*, apart from hypertonic sea-water (*cf.* next section), which apparently acts indirectly through its influence on metabolism. In numerous experiments with cytolytic compounds (*e.g.*, ether and chloroform), pure Na-salt solutions, ultra-violet radiation and the electric current, I have never obtained more than partial activation, whatever the duration of exposure.

[11] Made by mixing M/2 solutions of the following salts in the proportions by volume: 100 NaCl, 7.8 $MgCl_2$, 3.8 $MgSO_4$, 2.2 KCl, 2.0 $CaCl_2$.

apparently a summation effect resulting from a partial penetration of the external acid to the site of activation. The increased rate of activation observed in KCN-sea-water may similarly be regarded as a summation effect, due to the increased intracellular formation of acid under the conditions of asphyxiation. With regard to the reversal of hypersensitivity just described, it may be assumed that readmission of oxygen restores the egg to its original condition through the oxidative removal of the surplus of acid, as in the case of the lactic acid formed in muscle cells under asphyxia.

Experiments in which the eggs were placed for 19–20 minutes in sea-water saturated with pure oxygen from an oxygen-cylinder and then warmed to 32° in the same medium showed no significant difference from the control. (Results of three series: July 15, 16 and 18, 1930.) Apparently an increase of oxygen tension to 5 times the normal does not affect the rate of heat activation.

4. *Influence of O_2-lack and KCN on Activation by Hypertonic Sea-Water*

The case of hypertonic sea-water is peculiar. This agent, acting alone, is not so effective with starfish eggs as heat or fatty acid, and its rate of action is much slower. Nor is it so effective with these eggs as with *Arbacia* eggs. It is unusual to obtain 50 per cent of free-swimming blastulæ with hypertonic sea-water alone, although in a few experiments the proportion has reached 70 per cent or even higher. The most favorable procedure is to expose the eggs during the pre-maturational period to a mixture of 100 volumes sea-water *plus* 20 volumes 2.5 M NaCl for 1 1/2 to 2 hours (at 20–22°). Exposures during the post-maturational period are much less effective. In experiments with a series of hypertonic mixtures, consisting of 100 volumes sea-water *plus* (respectively) 10, 15, 20, 25 and 30 volumes 2.5 M NaCl, membrane-formation and blastulæ were obtained with all but the first and last. The effective osmotic gradient thus has a well-defined range; and within this range the optimal exposures show in general a decreasing duration with increasing concentration, as in Loeb's experiments with *Strongylocentrotus*.[12] The best results have been obtained with the 100 + 15 and 100 + 20 mixtures, corresponding to an increase in osmolar concentration of 50 to 60 per cent. The second of these solutions was used in most experiments.

Besides acting as a single parthenogenetic agent, hypertonic sea-water may also be used to supplement and complete a partial activation by heat or fatty acid (See Lillie, 1915, pp. 284 *seq.*).

A characteristic peculiarity in the mode of action of this agent is

[12] J. Loeb: Artificial Parthenogenesis and Fertilization, Chapter XI.

that the physiological change which it produces in the egg during the period of exposure is closely associated with the consumption of oxygen. In this respect the action of hypertonic sea-water offers a contrast to that of fatty acid and heat, both of which agents act independently of immediate oxygen consumption, besides being more uniform, rapid and complete in their activating effect.

Loeb showed many years ago that oxygen-free or KCN-containing hypertonic sea-water was ineffective as an after-treatment in sea-urchin eggs which had received the initial membrane-forming treatment.[13] In starfish eggs similar conditions are found, except that in order to deprive the hypertonic sea-water completely of its activating influence, it is necessary to expose the eggs previously for some time to cyanide or lack of oxygen. Eggs transferred from normal sea-water directly to oxygen-free or KCN-containing hypertonic sea-water always show some partial activation (*i.e.*, membrane-formation and occasionally a few blastulæ); while if they are kept first in oxygen-free (or KCN-containing) isotonic sea-water for 2 to 3 hours and then exposed to the oxygen-free (or KCN) hypertonic sea-water for the usual time (1 1/2 to 2 hours) the great majority show no signs of activation. If such eggs are then returned to sea-water and fertilized with sperm, a large proportion develop to larval stages, showing that they have remained essentially unchanged in their properties.

These conditions are illustrated by the experiments described in Tables IX and X.

TABLE IX

June 26, 1929. The eggs were placed, 40 minutes after removal, in (*A*) hypertonic sea-water of the composition 100 volumes sea-water *plus* 20 volumes 2.5M NaCl, and (*B*) in the same solution *plus* M/1000 KCN. Temperature 21° C. At intervals of 1½, 1¾ and 2 hours portions were transferred to normal sea-water.

Duration of exposure (*hours*)	Percentage of mature eggs forming blastulæ	
	A. Hypertonic sea-water	B. Hypertonic sea-water *plus* M/1000 KCN
1½	70–80	0
1¾	60–70	1–2
2	60–70	*ca.* 5

The eggs in this experiment were exceptionally responsive to hypertonic sea-water; it is in fact unusual to obtain any blastulæ after treatment with KCN-containing hypertonic sea-water. In a similar experiment on June 27 the eggs exposed to cyanide-free hypertonic sea-water

[13] *Loc. cit.*, Chapter XI.

for 1 1/2 hours at 23° formed 25 per cent of blastulæ, while with KCN present, although all formed membranes and many cleaved irregularly or fragmented, no blastulæ were obtained. Other experiments gave similar results.

The effect of placing in oxygen-free or KCN-containing sea-water for some time previous to the hypertonic treatment is shown in the following experiments (August 1, 1929).

TABLE X

Procedure	Result
A. Eggs exposed to hypertonic sea-water (100 + 20) alone, beginning 30 min. after removal, for 1½ and 1¾ hours (23°)	Activation in all; 25–35 per cent blastulæ
B. Eggs in O_2-free sea-water for 2 hours, beginning 30 min. after removal; thence to O_2-free hypertonic sea-water for 1½ and 1¾ hours	Great majority show no activation (no membranes); partial activation in a few
C. Eggs in sea-water + M/1000 KCN for 2 hours, beginning 35 minutes after removal; thence to KCN-containing (M/1000) hypertonic sea-water for 1½ and 1¾ hours	Similar to Experiment B. No activation except in small minority

A similar result was obtained in eight other experiments of the same kind, five with KCN-containing and three with oxygen-free hypertonic sea-water. It is evident that when the oxidative metabolism of the egg is suppressed, the abstraction of water by hypertonic media has little or no activating effect. The period of asphyxiation does not in itself deprive the eggs of responsiveness to hypertonic treatment, provided the oxygen is later restored. Eggs returned after 2 to 3 hours in KCN-containing sea-water to normal sea-water, washed thoroughly in sea-water and then exposed to hypertonic treatment show typical activation with production of blastulæ. As already shown, such eggs also retain their responsiveness to activation by heat and acid and to sperm-fertilization.

THEORETICAL

Assuming that the activation process consists in a regular sequence of interconnected physical and chemical processes, leading to some definite modification of the egg-system, we have to inquire how such a sequence can be initiated by a temporary slight rise of acidity. In general the experimental facts suggest that the first step in the chemical

sequence of activation is a transformation (*e.g.*, hydrolysis) of some compound present in the surface layer or cortical zone of the egg, this change leading directly or indirectly to the formation of some specific metabolic product ("activating substance"), the accumulation of which renders the egg capable of automatic development. Just how such a substance may be regarded as producing its effect is uncertain; it might contribute to special cell-structure, or alter already existing structure (*e.g.*, increase permeability), or it might be a catalyzer, or form some necessary link in the chain of structure-forming reactions. On the whole the last supposition seems the most likely one. Examples of the control of morphogenetic processes by special metabolic products are well known (*cf.*, the case of hormones); and they show at least that the hypothesis of an activating substance is consistent with the general facts of developmental physiology.

It seems unlikely that the primary or releasing reaction (or reactions) of activation can be widely different from those met with in other types of reactive cell. In general the basic chemical processes of protoplasm (as well as its basic chemical composition) show a well-defined uniformity throughout the range of living organisms. Thus we find that the carbohydrate metabolism of yeast cells resembles closely that of muscle cells, with certain divergences in detail; the utilization of oxygen by living cells depends on factors of a kind universally present in protoplasm (*cf.*, the heme and sulphydryl compounds); the course and end-products of protein metabolism are similar in their general character. Such considerations lead us to expect that the key reactions of activation may be simple and of a type already known.

The prompt arrest of the activation reaction when eggs are returned from acid to normal sea-water, and its equally prompt renewal on re-exposure to acid, are facts suggesting that the rise of acidity within the egg acts by releasing from combination some compound which is necessary for the specific reaction of activation, and that this releasing reaction is reversed on return to or toward neutrality, the compound re-entering combination and becoming again unavailable. The releasing reaction itself has apparently the character of a hydrolysis, and the dependent specific reaction which forms the activating substance is to be regarded as keeping pace with this hydrolysis.

Certain readily hydrolyzable compounds widely distributed in cells show a behavior of the general kind required by such a hypothesis. Of chief interest are the phosphate compounds or esters, especially the guanidin phosphates (phosphagens), which appear to be of special importance in the metabolic reactions associated with excitation in

muscle. The enzymatic hydrolysis of these compounds in muscle extracts is influenced by variations of acidity in a manner closely paralleling the characteristic variation in the rate of activation of eggs with concentration of acid. The creatin phosphate of vertebrate muscle extracts splits into creatin and phosphoric acid at a slightly acid reaction (pH = 6.4), and is resynthesized at slight alkalinity (pH = 8.5-9); the analogous arginin phosphate of invertebrates shows similar properties, but has a greater tendency to resynthesis at slightly alkaline or even neutral reaction.[14] The total phosphate content of starfish eggs is very similar to that of muscle cells (Page, 1927), and it is possible that in the activation and other reactions of these cells phosphate esters play a rôle of the same kind as in the analogous reactions of muscle and presumably other cells. Meyerhof has suggested that a special biological rôle of the guanidin compounds may consist in the binding and splitting off of phosphate groups (Meyerhof and Lohmann, 1928), a view in accordance with the general importance of phosphates in cell metabolism. The hypothesis that the splitting of phosphagen or some analogous compound is the primary chemical event in activation requires, however, further experimental testing from both the physiological and the biochemical sides.[15]

With regard to the activation by hypertonic sea-water, it is to be noted that abstraction of water from the egg means increase in concentration of egg-constituents, and it is possible that locally (*e.g.*, in the cortical region) this increase may be considerable. Conditions favorable to the synthesis of complex compounds would thus arise. The general theory of the dehydrolytic synthesis of complex molecules through the combination of a number of smaller molecules has recently been reviewed by Wasteneys and Borsook (1930, 1931) in relation to their work on the enzymatic synthesis of plasteins from the products of peptic hydrolysis. The authors show both theoretically and experimentally that increase in the concentration of such a digest may change the prevailing direction of the reversible reaction from a hydrolysis to a synthesis; and it is reasonable to assume that conditions of the same general kind exist in living cells. In the case of the starfish egg the inference would be that hypertonic sea-water acts by promoting the synthesis of some compound (*e.g.*, protein) which either is, or gives rise to, the activating substance.

[14] Meyerhof and Lohmann, *Biochem. Zeitschr.*, 196, 49, 1928. It might be objected that such reactions are too slow to account for the rapid course of the activation reaction in starfish eggs immersed in acid sea-water, and its rapid arrest on return to normal sea-water; but it is well-known that many enzymatic reactions which are slow *in vitro* proceed rapidly in the living cell.

[15] *E.g.*, it would be important to determine if the free phosphate is increased in the egg during activation, as in the stimulation of muscle and nerve.

It is significant that the formation of the activating substance appears to be promoted by two quite different external changes of condition, hypertonicity and moderate acidity. As already described, hypertonic activation proceeds relatively slowly as compared with acid activation and is dependent on oxygen consumption. The essential problem is why dehydration combined with oxidation should lead to the production of the same substance as slight local increase of acidity. Experimentally we find that the two parthenogenetic procedures may act additively or supplement each other; according to the present hypothesis, however, they influence metabolism in different ways, the one promoting synthesis of a complex compound, the other releasing hydrolytically some relatively simple compound which enters further combination. The fact that both procedures have the same final physiological effect suggests that the activating substance is formed by the combination of the two products. In such a case, according to the mass action rule, increase in the concentration of the one product would offset deficiency in the other; *i.e.*, activation would result from a sufficient increase in either compound, provided some of the other were available. This would explain why either acid or hypertonic sea-water acting alone may cause complete activation, since both compounds may be assumed to be present in low concentration in the resting egg. Whether the additive relations between the two parthenogenetic procedures conform to such a hypothesis in detail can be determined only by further experiment.

Summary

1. Suppression of oxygen-consumption in freshly removed unfertilized starfish eggs (by exposure to cyanide-containing or oxygen-free sea-water) prolongs by several hours the period during which they remain responsive to artificial activation by heat, acid, or hypertonic sea-water (as well as to sperm fertilization). The possible interval between a first partial and a second completing activation may be similarly prolonged.

2. During the exposure of the eggs to these asphyxiating conditions their susceptibility to activation by heat or acid, as indicated by the effective durations of exposure, undergoes a progressive increase.

3. Eggs kept for some hours in cyanide-containing or oxygen-free sea-water and then exposed to acid or heat while immersed in these media show normal activation. On the other hand, a similar suppression of oxygen consumption prevents activation by hypertonic sea-water.

4. It is suggested that the activation by hypertonic sea-water de-

pends on the increased intracellular production, by dehydrolytic synthesis, of some complex specific compound (*e.g.*, protein); while in the anaërobic activation by acid (or heat) the critical change is a hydrolysis (*e.g.*, of a phosphagen compound), yielding a product which combines with the complex compound to form the specific activating substance. The accumulation of this substance to a certain definite level determines activation. Two metabolic processes, respectively aërobic and anaërobic, would thus coöperate in activation. The fact that either hypertonic sea-water or acid (or heat), acting alone, can produce the same physiological end-effect, complete activation, is shown to be consistent with this hypothesis.

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